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Three strategies of transgenic manipulation for crop improvement

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Heterologous expression of exogenous genes, overexpression of endogenous genes, and suppressed expression of undesirable genes are the three strategies of transgenic manipulation for crop improvement. Up to 2020, most (227) of the singular transgenic events (265) of crops approved for commercial release worldwide have been developed by the first strategy. Thirty-eight of them have been transformed by synthetic sequences transcribing antisense or double-stranded RNAs and three by mutated copies for suppressed expression of undesirable genes (the third strategy). By the first and the third strategies, hundreds of transgenic events and thousands of varieties with significant improvement of resistance to herbicides and pesticides, as well as nutritional quality, have been developed and approved for commercial release. Their application has significantly decreased the use of synthetic pesticides and the cost of crop production and increased the yield of crops and the benefits to farmers. However, almost all the events overexpressing endogenous genes remain at the testing stage, except one for fertility restoration and another for pyramiding herbicide tolerance. The novel functions conferred by the heterologously expressing exogenous genes under the control of constitutive promoters are usually absent in the recipient crops themselves or perform in different pathways. However, the endogenous proteins encoded by the overexpressing endogenous genes are regulated in complex networks with functionally redundant and replaceable pathways and are difficult to confer the desirable phenotypes significantly. It is concluded that heterologous expression of exogenous genes and suppressed expression by RNA interference and clustered regularly interspaced short palindromic repeats-cas (CRISPR/Cas) of undesirable genes are superior to the overexpression of endogenous genes for transgenic improvement of crops.

KEYWORDS

commercial release, heterologous expression, endogenous gene, exogenous gene, overexpression, suppressing expression, transgenic crop

Introduction

Breakthrough of crop improvement

To meet the food demand of the booming world population, the comprehensive requirements for yield, quality, and adaptability of crop cultivars are becoming more and more urgent (Barrett, 2021). Due to the limitation of genetic variation within nature or mutagenized populations of sexually compatible species, conventional approaches to crop improvement, such as systematic breeding, crossing breeding, and heterosis utilization, are laborious and time-consuming. However, transgenic technology surmounts hybridization barriers and utilizes the desirable genes from genetically distant species, to realize molecular design breeding to a certain extent (Raymond Park et al., 2011; Kamthan et al., 2016). It is thought that transgenic technology has been a revolutionary impact on crop improvement as a second Green Revolution, greatly improving the yield, quality, and adaptability of crops and making an important contribution to ensuring food security (Eckardt et al., 2009; Farre et al., 2010; Kamthan et al., 2016). Transgenic cultivars of crops are developed by cloning desirable genes, constructing expression vectors, genetic transformation of recipient crops, screening and identification of transformed lines, so as to improve the original undesirable traits or endow them with new beneficial traits (Raymond Park et al., 2011; Kamthan et al., 2016). In addition, transgenic technology is also used to modify or knock out the undesirable genes of crops to change their genetic characteristics and obtain the desirable phenotypes (Georges and Ray, 2017). After a safety assessment, transgenic cultivars with significant improvement in yield, quality, or adaptability are approved for commercial release.

The rapid increase of transgenic crops

The first transgenic plants were developed about four decades ago with traits like antibiotic and insect resistances (Bevan et al., 1983; Fraley et al., 1983; Herrera-Estrella et al., 1983; Murai et al., 1983). Since the approval of the transgenic tomato variety with delayed maturation for commercial release by the food and drug administration (FDA) after stringent scientific scrutiny and credible safety assessment in 1994 (Klee, 1993; Parrott et al., 2010; Giraldo et al., 2019), transgenic crops, like insect resistant cotton and maize, herbicide-resistant soybean and canola, have received marketing approval one after another (Padgett et al., 1995; Schuler et al., 1998; Bates et al., 2005), and transgenic technology has increased the pace of crop improvement to meet the requirements of biotic and abiotic resistance, higher yield, and nutritional value (Raymond Park et al., 2011). According to the survey carried out by the International

Service for the Acquisition of Agri-Biotech Applications (ISAAA, 2022), the commercialized acreage of transgenic crops has straightly increased to 176.85 million hectares in the world by 2021 (Figure 1). This acreage distributes in more than 30 countries in all, including industrial and developing countries. Great profitability has been achieved by increasing yield and reducing input in pesticides, labor, and machinery (Naranjo, 2011; Raymond Park et al., 2011; Smyth et al., 2014).

Achievements of three transgenic strategies

Three transgenic strategies

Heterologous expression of exogenous genes, overexpression of endogenous genes, and suppressed expression of undesirable genes are the three strategies of transgenic manipulation for crop improvement. The first strategy is the transformation of crops by exogenous genes from genetically distant species. The second strategy is also proposed as cisgenesis and intragenesis by the transformation of endogenous genes from the same species or homologous genes from sexually compatible species, respectively, if no foreign DNA such as selectable marker gene and vector-backbone sequence is introduced into the cisgenes or intragenes by *in vitro* mutagenesis or other means (Rommens et al., 2007; Schouten and Jacobsen, 2008; Jacobsen and Schouten, 2009). These alternative concepts mitigate the public concerns about the biosafety of genetically modified (GM) crops developed by transgenesis (Schouten et al., 2006). The third strategy is to suppress the expression of undesirable endogenous or pathogenic and pest genes by the introduction of synthetic sequences transcribing antisense or double-stranded RNAs (Mamta and Rajam, 2017; Zhang J. et al., 2017; Hernández-Soto and Chacón-Cerdas, 2021), or to knock out them by clustered regularly interspaced short palindromic repeats-cas (CRISPR/Cas) technology as well as some other more complicated technologies of genome editing such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and mega-nucleases (MNs) (Gaj et al., 2013; Jaganathan et al., 2018; Langner et al., 2018; Gao, 2021; Rasheed et al., 2021; Samaras et al., 2021; Sharma and Vakhlu, 2021; Turnbull et al., 2021). Especially CRISPR/Cas technology, as a simple, easy, and cost-effective tool of precise and straightforward genome-wide gene editing, has been developed as a potential strategy for crop improvement and helped much to mitigate the public's negative perception of GM food crops (Naeem et al., 2020; Gao, 2021; Rasheed et al., 2021).

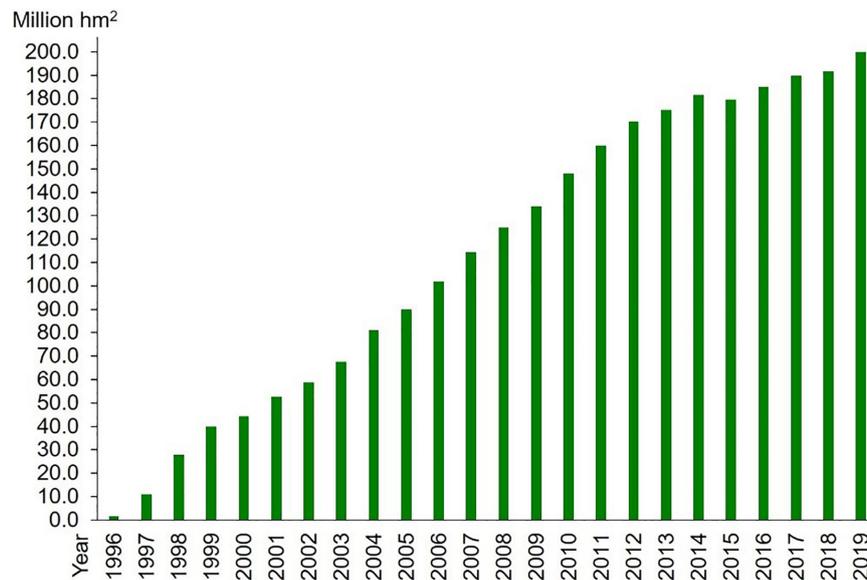


FIGURE 1

Commercialized acreage of transgenic crops.

Transgenic events approved for commercial release

According to the survey carried out by ISAAA (2022), most (227) of the singular transgenic events (265) approved for commercial release worldwide were developed by the first strategy: 210 by bacterial genes (Stalker et al., 1988; Ye et al., 2000; Paine et al., 2005; Castiglioni et al., 2008; Napier et al., 2019), 8 by exogenous genes from mold, algae, fungus, and yeast (Knutzon et al., 1998; Napier et al., 2019; Kinney et al., 2022), 5 by exogenous genes from sexually incompatible plant species (Song et al., 2003; Takagi et al., 2005; Preuss et al., 2012; Rice et al., 2014), and 2 by the mutant copies of the endogenous genes for enhancing herbicide tolerance (McNaughton et al., 2008; EFSA Panel on Genetically Modified Organisms [GMO] et al., 2018; Karthik et al., 2020), respectively (Table 1). Thirty-eight events were developed by the third strategy and transformed by synthetic sequences transcribing antisense or double-stranded RNAs for suppressed expression of undesirable endogenous genes of pathogens, pest insects, and recipient crops themselves (Chen et al., 2003; Davis and Ying, 2004; Tennant et al., 2005; Otani et al., 2007; Ilardi and Nicola-Negri, 2011; Aragao et al., 2013; Ramaseshadri et al., 2013; Carvalho et al., 2015; Orbegozo et al., 2016; Borah et al., 2018; Wu et al., 2018; Callahan et al., 2019; Chiozza et al., 2020). Only one event was transformed by endogenous genes for restoring male fertility (Unger et al., 2002) and another event for pyramiding herbicide tolerance. Of course, antibiotic or herbicide-resistant genes from bacteria were also introduced into almost all of these events as selection markers of transformant screening (Demaneche et al., 2008). By

CRISPR/Cas technology, several events have developed, skipped regulation of government, and entered the market because of their safety assurance, and some more events have been in the pipeline of safety assessment (Hartung and Schiemann, 2014; Waltz, 2016a,b; Wolt et al., 2016; Faure and Napier, 2018; Jaganathan et al., 2018; Langner et al., 2018; Samaras et al., 2021).

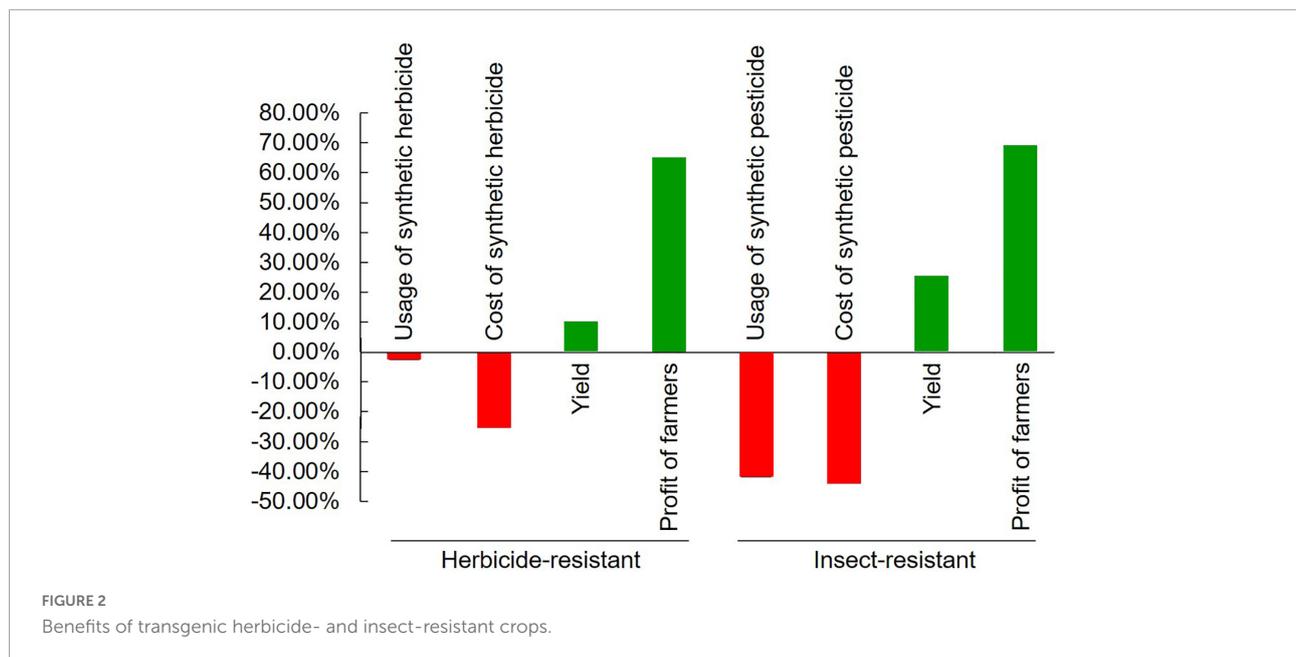
Great achievements of heterologous expression of exogenous genes

Weeds burden plant growth as they compete for space, sunlight, and soil nutrients leading to 25–80% yield losses (Awan et al., 2015; Ramachandra et al., 2016). The application of synthetic herbicides is an effective approach to control weeds but causes great waste of resources, and serious problems of environmental pollution and food safety (Vandenberg et al., 2017; Panthi et al., 2019). GM varieties transformed by herbicide-resistant genes give the feasibility to combat weeds and thus help in the safety of the crops without major yield losses (Benbrook, 2016; Ramachandra et al., 2016). Glyphosate [*N*-(phosphonomethyl) glycine] is a widely used broad-spectrum herbicide that controls weeds by inhibiting the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme and interfering with the shikimate biosynthesis pathway (Funke et al., 2006). However, this non-selective herbicide also damages the crops. The current strategy is to improve glyphosate resistance in crops by the transformation of the EPSPS, *GAT*, and *Goxv* genes that encode for an insensitive

TABLE 1 Singular transgenic events approved for commercial release.

GM crop	GM trait	Event	Bacterial	Distant species	Plant			RNAi
					Exogenous	Mutant	Endogenous	
Alfalfa	Herbicide tolerance	2	2					
	Quality improvement	4						4
Bean	Viral resistance	1						1
Canola	Herbicide tolerance	23	23					
	Quality improvement	9	2	7	1			
Cotton	Herbicide tolerance	28	28		3			
	Insect resistance	26	27		1			
	Quality improvement	1						1
Cowpea	Insect resistance	1	1					
Eggplant	Insect resistance	1	1					
Flax	Herbicide tolerance	1	1					
Maize	Herbicide tolerance	18	16			3	1	
	Insect resistance	7	7					
	Herbicide/insect tolerance	18	18					
	Drought tolerance	1	1					
	Quality improvement	5	3	1	1			
	Male sterility	3	3					
Melon	Fertility restoration	1					1	
	Delayed maturation	2	2					
Soybean	Herbicide tolerance	18	17		1	3		
	Insect resistance	2	2					
	Herbicide/insect tolerance	2	2					
	Herbicide tolerance/growth regulation	1			1			
	Drought tolerance	1			1			
Papaya	Quality improvement	4			1	2		1
	Viral resistance	4						4
Plum	Viral resistance	1						1
Potato	Herbicide tolerance	4	4					
	Insect resistance	29	29					
	Viral resistance	15						15
Rice	Herbicide tolerance	3	3					
	Insect resistance	3	3					
	Quality improvement	2	2		1		1	
Squash	Viral resistance	2						2
Sugar beet	Herbicide tolerance	3	3					
Sugarcane	Insect resistance	3	3					
	Drought tolerance	3	3					
Sweet pepper	Viral resistance	1						1
Tobacco	Herbicide tolerance	1	1					
	Quality improvement	1						1
Tomato	Insect resistance	1	1					
	Viral resistance	1						1
	Delayed maturation	9	2			1		6
Wheat	Herbicide tolerance	1	1					
Total		265	210	8	5 (+6)	3 (+6)	2 (+1)	38
Technological strategy			First				Second	Third
			227 (+12)				2 (+1)	38

Distant species include mold, algae, fungus, and yeast. RNAi was triggered by antisense and double-stranded RNA described by the introduced synthetic DNA sequence. The gray background indicates the stacked genes. The numbers of the events introduced stacked genes were put in the brackets. All the information is from ISAAA (2022).



form of EPSPS of acetyltransferase and glyphosate oxidase, respectively (Block et al., 1987; Padgett et al., 1995; Castle et al., 2004; McNaughton et al., 2008; Yan et al., 2011; Awan et al., 2015; Chhapekar et al., 2015; Guo et al., 2015; Liang et al., 2017; Zhang X. B. et al., 2017; Yi et al., 2018). The other widely used synthetic herbicides are glufosinate and bialaphos, which inhibit the activity of glutamine synthetase and thus block all nitrogen assimilation into the plant. Tolerant varieties are developed by the transformation of the genes *PAT* and *Bar* that encode for phosphinothricin acetyltransferase (*PAT*), which detoxifies the herbicides (Hérouet et al., 2005; Herman et al., 2013; Cegielska-Taras et al., 2008; EFSA Panel on Genetically Modified Organisms [GMO] et al., 2020a,b). Bromoxynil (3,5-dibromo 4-hydroxybenzotrile) and other oxynil herbicides inhibit photosynthesis by blocking electron flow during the light reaction, causing the production of reactive oxygen species (ROS), destruction of cell membranes, inhibition of chlorophyll formation and death. Resistance is conferred by the *bxn* gene that encodes a nitrilase enzyme that detoxifies the herbicide (Stalker et al., 1988). All these herbicide-resistant genes are derived from various species of soil bacteria *Agrobacterium*, *Pseudomonas*, *Streptomyces*, *Bacillus*, *Ochrobactrum*, and *Klebsiella* (Block et al., 1987; Thompson et al., 1987; Stalker et al., 1988; Castle et al., 2004; Siehl et al., 2007; Yan et al., 2011). Only very few of them are transformed by an herbicide-resistant mutant of exogenous or endogenous plant *EPSPS* genes (Table 1; McNaughton et al., 2008; EFSA Panel on Genetically Modified Organisms [GMO] et al., 2018; Karthik et al., 2020). From these transgenic events, 4 alfalfa, 52 canola, 65 cotton, 345 maize, 4 potato, 3 rice, 52 soybean, 2 sugar beet, 1 tobacco, and 1 wheat cultivars have been developed and approved for commercial

release¹. A meta-analysis shows that the application of these transgenic herbicide-resistant varieties reduces the use of synthetic herbicide by 2.43% and the cost of herbicide by 25.29%, increases the yield of crops by 9.29%, and benefits farmers by 64.29% (Figure 2; Klumper and Qaim, 2014). However, great attention should be paid to the potential environment and agronomic impact caused by intraspecific gene flow from the transgenic herbicide-resistant varieties to weeds or their non-GM counterparts, especially in partly cross-pollinated canola and other crops of the crucifer family (Légère, 2005; Bonny, 2016; Sharkey et al., 2021). This problem should be seriously solved by the choice of suitable cultivars and certified seeds as well as by weed and soil management (Devos et al., 2004; Hüskén and Dietz-Pfeilstetter, 2007). The potential CRISPR/Cas technology should play important role in combating this problem (Hussain et al., 2021).

Insect pests used to be the major biotic stress that caused a serious reduction in crop productivity globally (Oerke, 2006; Douglas, 2018). The extensive application of chemical pesticides not only increased production costs but also caused severe environmental pollution (Aktar et al., 2009; Birkett and Pickett, 2014). Transgenic insect-resistant crops (mainly cotton, maize, and soybean) have made a beneficial and eco-friendly impact on crop production (Matten and Reynolds, 2003; Gatehouse et al., 2011; Blanco, 2012; Rocha-Munive et al., 2018). The majority (68) of the transgenic insect-resistant events (70) are developed by heterologous expression of the insecticidal genes *Cry* (δ -endotoxin) from different strains of soil bacterium *Bacillus thuringiensis* (Ghareyazie et al., 1997), except for 1 maize, 2

¹ <https://www.isaaa.org/gmapprovaldatabase>

poplar, and 2 cotton events simultaneously transformed by vegetative insecticidal protein genes *vip3*, *CpTI*, and *API*, as well as double-stranded RNA transcript of gene *Snf7* from western corn rootworm (*Diabrotica virgifera*), respectively, for pyramiding broad resistance (Table 1; Xie et al., 1997; Hu et al., 2001; Cui et al., 2011; Ramaseshadri et al., 2013). From these events, 59 cotton, 1 cowpea, 1 eggplant, 341 maize, 3 poplar, 30 potato, 3 rice, 6 soybean, 3 sugarcane, and 1 tomato cultivars resistant to lepidopteran (246), coleopteran (156), hemipteran (1), as well as multiple insects (36), respectively, have been developed and approved for commercial release (ISAAA, 2022). The majority of published studies on transgenic cotton performance have documented the decrease in insecticide application, and the increase in yield and benefits in developed and developing countries (Showalter et al., 2009). A meta-analysis shows that the application of insecticidal transgenic crops has decreased the use of synthetic pesticides by 41.67% and the cost of pesticides by 43.43%, increased the yield of crops by 24.85%, and benefited farmers by 68.78% (Figure 2; Klumper and Qaim, 2014). This analysis is confirmed by an actual survey on the application of transgenic insect-resistant cotton in China (Pray et al., 2001).

Vitamin A deficiency is common in children in developing countries who rely on rice as a staple food (Cabezuelo et al., 2020). Transgenic “Golden” rice and potato heterologously expressing three bacterial genes (*CrtB*, *CrtI*, and *CrtY*) encoding phytoene synthase, phytoene desaturase, and lycopene β -cyclase, respectively, for enhancing β -carotene synthesis and accumulation have been developed and approved for commercial release, and directly used as food in the United States, Canada, New Zealand, Australia, Nigeria, Kenya, and the Philippines (Table 1; Ye et al., 2000; Paine et al., 2005; Chitchumroonchokchai et al., 2017; Napier et al., 2019), although endogenous orthologs of these genes are present in the genomes of rice and potato (Thorup et al., 2000; Koc et al., 2015; Banakar et al., 2020; Yang et al., 2021).

Canola (*Brassica napus*) is a high-yield oil crop. However, the quality of its oil is required to be improved to decrease the high proportion of saturated fatty acids. Genetic modification by introducing desaturase genes and desaturase-related genes is an effective approach (Napier et al., 2019). Although these genes are present in the genome of canola itself (Xue et al., 2018), all the nine events approved for commercial release (ISAAA, 2022) have been transformed by stacked exogenous genes from mold, algae, fungus, and yeast (Table 1; Knutzon et al., 1998; Napier et al., 2019; Kinney et al., 2022).

Overexpression of endogenous genes remains at the testing stage

By the strategy of overexpression of endogenous genes, numerous transgenic events have also been developed and

their target phenotypes reported to be enhanced. For example, drought is one of the most significant constraints on crop production (Cohen et al., 2021). A lot of literatures have documented the improvement of drought tolerance of transgenic crops overexpressing endogenous or homologous genes encoding function proteins related to osmotic protectants, membrane stabilization, detoxification and transport, such as SOD (superoxide dismutase), VP1 (vacuolar proton-pumping pyrophosphatase), BADH (betaine aldehyde dehydrogenase), P5CS (Δ 1-pyrroline-5-carboxylate synthetase), LEA (late embryogenesis-abundant proteins), and FER (ferritin), as well as transcription factors and signaling molecules, such as DREB (dehydration-responsive element binding), ABF [abscisic acid (ABA)-responsive elements binding factor], AP2/ERF (ethylene response factor), bZIP (basic leucine zipper), MYB, MYC, NAC (no apical meristem), ZFP (zinc finger protein), HD-Zip (homeodomain-leucine zipper), WRKY, NF (nuclear factor), HRD, and HYR (higher yield rice) (Kasuga et al., 1999; Nelson et al., 2007; Xiao et al., 2007; Century et al., 2008; Huang et al., 2009; Wu et al., 2009; Tran et al., 2010; Varshney et al., 2011; Xue et al., 2011; Datta et al., 2012; Schilling et al., 2017; Bi et al., 2018; Gao et al., 2018; Yang et al., 2018; Sarkar et al., 2019; Wei et al., 2019). However, the vast majority of these efforts have still remained at the testing stage. Of the five singular transgenic events approved for commercial release (Table 1; ISAAA, 2022), one has been transformed by the cold shock protein gene *CspB* from *B. subtilis* and three by the choline dehydrogenase genes *BetA* from *Escherichia coli* and *Rhizobium meliloti*, respectively, although the cold shock proteins are also found in many eukaryotic species (Castiglioni et al., 2008; Tollefson, 2011). Only one has been transformed by the exogenous transcription factor gene *Hahb-4* from sexually incompatible sunflower (*Helianthus annuus*) (Ribichich et al., 2020).

RNA interference is effective for suppressing expression of undesirable genes

Plant diseases reduce crop yield and quality and bring huge economic losses (Gimenez et al., 2018). Transgenic technology has been employed to battle against a wide range of plant pathogens (Wally and Punja, 2010; Kamthan et al., 2016). Similar to drought tolerance, the vast majority of the transgenic events overexpressing endogenous disease-resistant genes or homologous disease-resistant genes from sexually incompatible species remain at the testing stage (Anand et al., 2003; Zhao et al., 2005; Yang et al., 2008; Zhou et al., 2009). Twenty-five of the 29 approved events have been developed by the third strategy and transformed with synthetic DNA sequences to transcribe antisense or double-stranded RNAs for the interference of disease viruses (Chen et al., 2003; Davis and Ying, 2004; Tennant et al., 2005; Aragao et al., 2013; Carvalho et al., 2015;

Borah et al., 2018; Wu et al., 2018; Callahan et al., 2019; Chiozza et al., 2020), and only the other four potato events are transformed with exogenous genes of pathogenesis-related proteins from distant species of the nightshade family (*Solanum bulbocastanum* and *Solanum venturii*) (Table 1; Halterman et al., 2008; Foster et al., 2009). RNA interference (RNAi) triggered by antisense or double-stranded RNAs described by transformed synthetic DNA sequences is a versatile, effective, safe, and eco-friendly technology for crop protection against viruses and other pathogens as well as insect pests, and delaying maturation of fruits with positive economic, environmental, and human health implications (Klee, 1993; Taning et al., 2020; Giudice et al., 2021; Hernández-Soto and Chacón-Cerdas, 2021).

Application of clustered regularly interspaced short palindromic repeats/Cas9 for crop improvement

Since its first discovery in *E. coli* (Ishino et al., 1987), CRISPR/Cas has been developed as a simple, easy, and cost-effective tool for precise and straightforward genome-wide gene editing (Gaj et al., 2013; Tang et al., 2017; Molla and Yang, 2019; Molla et al., 2020, 2021; Sharma and Vakhlu, 2021). Unlike ZFNs, TALENs, and MNs, CRISPR/Cas could be used to modify any genomic sequences, thereby providing a simple, easy, and cost-effective means of precise and straightforward genome-wide gene editing (Gaj et al., 2013; Tang et al., 2017; Gao, 2021; Leibowitz et al., 2021; Rasheed et al., 2021; Sharma and Vakhlu, 2021; Turnbull et al., 2021). However, In the beginning, most of the studies focused more on the concept proofing of the CRISPR/Cas system (Nekrasov et al., 2013; Shan et al., 2013; Xie and Yang, 2013; Zhang et al., 2014; Lawrenson et al., 2015; Xie et al., 2015; Hu et al., 2016; Malnoy et al., 2016; Zhu et al., 2016; Li et al., 2017; Shimatani et al., 2017). Although many attempts have been made to improve the yield, quality, and biotic and abiotic tolerance of different crops (Xie and Yang, 2013; Liang et al., 2014; Wang et al., 2014, 2021; Zhang et al., 2014, 2018, 2020; Fang and Tyler, 2016; Li et al., 2016, 2017, 2020; Zaidi et al., 2016; Zhu et al., 2016; Shi et al., 2017; Shimatani et al., 2017; Yang et al., 2017; Kim et al., 2018; Okuzaki et al., 2018; Shen et al., 2018; Usman et al., 2020, 2021; Zeng et al., 2020; Monsur et al., 2021), only very few events have been in the pipeline of safety assessment up to now (Waltz, 2016a,b). In recent years, several techniques, such as high attractive sgRNA, high fidelity Cas9, and transformant screening, have been developed to reduce the probable off-target effects caused by the imperfect matches with gRNA and the unpredictable efficiency among different DNA target sites and PAM (Naem et al., 2020; Leibowitz et al., 2021). CRISPR/Cas has been improved as the most promising tool for crop improvement (Gao, 2021; Rasheed et al., 2021; Turnbull et al., 2021) and applied to improve yield, quality, and biotic and abiotic tolerance (Wang et al., 2014, 2021; Fang and Tyler, 2016;

Li et al., 2016, 2017, 2020; Malnoy et al., 2016; Zaidi et al., 2016; Braatz et al., 2017; Shi et al., 2017; Yang et al., 2017; Okuzaki et al., 2018; Shen et al., 2018; Zhang et al., 2018, 2020; Usman et al., 2020, 2021; Zeng et al., 2020; Monsur et al., 2021). Up to now, several events have been developed and skipped regulation of government and entered the market because of their safety assurance, and some more events have been in the pipeline of safety assessment (Hartung and Schiemann, 2014; Waltz, 2016a,b; Wolt et al., 2016; Faure and Napier, 2018; Jaganathan et al., 2018; Langner et al., 2018; Samaras et al., 2021).

Superiority of heterologous expression of exogenous genes

Novel functions conferred by exogenous genes are less regulated by endogenous pathways

The functions conferred by the heterologously expressing exogenous genes are usually novel in the recipient crops themselves, such as herbicide and insect resistance in the released events of transgenic cotton, maize, soybean, and alfalfa (Cui et al., 2011; Awan et al., 2015; Chhapekar et al., 2015; Guo et al., 2015; Liang et al., 2017; Yi et al., 2018), or perform in different pathways, such as the synthesis of β -carotene and unsaturated fatty acids in the released events of transgenic rice and canola (Knutzon et al., 1998; Ye et al., 2000; Paine et al., 2005; Wan et al., 2017). In the vast majority of the above transgenic events, the exogenous genes are promoted by the constitutive promoters (Paine et al., 2005; Cui et al., 2011; Awan et al., 2015; Chhapekar et al., 2015; Guo et al., 2015; Liang et al., 2017; Zhang J. et al., 2017; Yi et al., 2018). Therefore, their expression is usually not regulated on the transcriptional level, although some other factors such as genetic background and growth stage of the recipient cultivars, and environmental conditions may affect their expression by several folds (Adamczyk and Meredith, 2004; Showalter et al., 2009; Poongothai et al., 2010; Chen et al., 2019). In case of the transgenic events are developed by the introduction of distant prokaryotic genes, possible codon usage bias is usually overcome by codon optimization of the transgene sequences (Siehl et al., 2007; Liu, 2009; Yan et al., 2011; Chhapekar et al., 2015; Liang et al., 2017; Yi et al., 2018). The investigations in transgenic insecticidal cotton ($r = 0.762$, $p < 0.001$) and rice ($r = 0.742$, $p < 0.01$) show that the accumulation of the Cry protein in leaves is non-linearly correlated with the heterologous transcription levels of the exogenous Cry genes, although varying with growth and development (Adamczyk and Sumerford, 2001; Adamczyk et al., 2001, 2009; Zhang et al., 2016). Adamczyk and Meredith (2004) suggest that a small number of endogenous genetic factors control the accumulation of the Cry protein in transgenic

cotton. Therefore, these exogenous proteins usually diverge from the endogenous metabolism pathways of the recipient crops (Sanahuja et al., 2011; Palma et al., 2014; Melo et al., 2016). The resistance conferred by these prokaryotic toxins is not easy to be overcome by the evolution of the pest insects. Instead, it can be augmented and complemented by pyramiding broad resistance expressing different combinations of insecticidal genes with different insecticidal mechanisms, or silencing the housekeeping genes of pest insects by RNAi and CRISPR/Cas technologies (Bates et al., 2005; Carriere et al., 2015; Katta et al., 2020; Talakayala et al., 2020). In agricultural practice, integrated pest management is still necessary to control the non-targeted pests of Cry protein (Naranjo, 2011; Downes et al., 2017).

Endogenous proteins are regulated in complex networks

Almost all biochemical reactions are reversible, and most of them are regulated in complex networks (Alberty, 2002; Fiehn and Weckwerth, 2003). One of the fundamental predictions of metabolic control theory is that, while any step in a pathway can be made to control flux if the step is blocked, increasing the activity of an enzyme may not necessarily result in increased flux through the reaction it catalyzes (Kacser and Burns, 1995). For example, plant lipids are a complex mixture of several hundreds of triacylglycerol fatty acids (Dormann, 2021). The relative content and saturation degree of these fatty acids determine the functional, sensory and nutritional value of the oil. Their synthesis metabolism is well regulated in a complex network among alternative pathways across multiple subcellular compartments (King et al., 2015; Chapman and Feussner, 2016; Haslam et al., 2016; Wan et al., 2017). In soybean cotyledon, the activity of stearyl-acyl carrier protein (ACP) desaturase is excess and thus overexpression of an endogenous stearyl-ACP desaturase gene does not result in any changes in the accumulation and proportion of fatty acids (Kinney, 1996; Voelker and Kinney, 2001). However, the synthesis pathway can be rationally modified by the introduction of exogenous genes from distant species to produce novel fatty acids of high value that are absent or typically found at low levels in oil

crops (Voelker and Kinney, 2001; Thelen and Ohlrogge, 2002; Haslam et al., 2016). All the four transgenic canola events approved for commercial release (ISAAA, 2022) are introduced with stacked exogenous genes from distant species of bacterium, mold, yeast, algae, fungus, moss, and amastigote (Knutzon et al., 1998; Napier et al., 2019).

Another example is the transgenic improvement of drought tolerance. Drought tolerance of plants is mediated by signal transduction ionic and osmotic homeostasis, detoxification, and growth pathways. The ionic aspect is signaled *via* the SOS pathway where a calcium-responsive SOS3-SOS2 protein kinase complex controls the expression and activity of ion transporters such as SOS1. Osmotic stress activates several protein kinases including mitogen-activated kinases, which may mediate osmotic homeostasis and/or detoxification responses. A number of phospholipid systems are activated by osmotic stress, generating a diverse array of messenger molecules, some of which may function upstream of the osmotic stress-activated protein kinases. The phytohormone ABA plays a crucial role in plant growth and development, especially in response to abiotic stresses. The endogenous ABA level is controlled by complex regulatory mechanisms involving biosynthesis, catabolism, transport, and signal transduction pathways. This complex regulatory network responds to abiotic stresses at multiple levels, including transcription, translation, and post-translational regulation of tolerance-related genes (Zhu, 2002, 2016; Cutler et al., 2010; Dong et al., 2015). After perception by proteins of the PYR/PYL/RCAR family, the ABA-bound PYR/PYL/RCARs interact with clade A protein phosphatases type 2Cs (PP2Cs) and prevent them from inhibiting the sucrose non-fermenting 1-related protein kinase 2s (SnRK2s) (Fujii et al., 2009; Ma et al., 2009; Park et al., 2009). The activated SnRK2s induce ABA-responsive gene expression by phosphorylating transcription factors such as ABA-responsive element-binding factors (ABFs) and regulate many other processes through phosphorylating other substrates (Umezawa et al., 2013; Wang et al., 2013). Conversely, recent researches show that the PYR/PYL/RCAR receptors themselves are regulated by other pathways (Yu et al., 2020), and even they repress the activity of ABA-independent SnRK2s (Zhao et al., 2018). In the maize genome, there are 13, 16, and 11 members in the ZmPYL family, clade A of

TABLE 2 Advantages and limitations of three transgenic strategies.

	The first strategy	The second strategy	The third strategy	
			RNAi	CRISPR/Cas
Transformed sequence	Genetically distant genes	Endogenous genes	Antisense or double-stranded DNAs	CRISPR and Cas9
Phenotype	Conferring novel phenotypes	Enhancing desirable phenotypes	Suppressing undesirable phenotypes	Modifying phenotypes
Regulation	Novel proteins functioning in diverse pathways	Endogenous proteins regulated in complex networks	Suppressing synthesis of target proteins	Suppressing or modifying synthesis of target proteins
Approved event	227	2	38	1

the ZmPP2C family, and the ZmSnRK2 family, respectively. Therefore, the possible alternative pathways have as many as 2288 ($13 \times 16 \times 11$) from the ZmPYLs through the ZmPP2Cs to the ZmSnRK2s (Wang et al., 2018). Most of these pathways are functionally redundant and replaceable, so overexpression of the endogenous genes in any of these 2288 pathways is difficult to cause a significant improvement for tolerant phenotypes (Zhu, 2016). Their transformed lines remain at the testing stage (Hu et al., 2010; Xiang et al., 2017; Bhatnagar et al., 2020; Wang et al., 2020). Moreover, transformation may generate completely new interactions between the transgenes making them function differently from what is expected. Possible negative interactions between the desired phenotypes and other traits should be accounted (Khan et al., 2019). For example, the barley and wheat transformants of wheat transcription factor gene *HD-ZipI* (homeodomain-leucine zipper) and the tobacco transformant of maize DREB gene *ZmDREB4.1* showed improved resistance to drought but also exhibited an undesirable reduction of biomass and yield (Kovalchuk et al., 2016; Li et al., 2018; Yang et al., 2018). In many studies, the tolerance of transgenic events was evaluated by pot experiments in a greenhouse, which was different from the response of plants to water deficit in a gradual manner under natural conditions (Ortiz et al., 2007; Passioura, 2012; Pierre et al., 2012). A review by Cattivelli et al. (2008) of improvements in drought tolerance considers the new insights into the complexity of plant mechanisms enabled by genomics, but there is still a large gap between yields in optimal and stress conditions.

Conclusion

- (1) The vast majority of the singular transgenic events approved for commercial release worldwide are transformed by genetically distant exogenous genes. The heterologous expression of these genes improves the resistance of crops to herbicides and pesticides, as well as the nutritional quality. Their application significantly decreases the use of synthetic herbicides and pesticides, reduces cost, increases the yield of crops, and benefits farmers. The novel functions conferred by these genes under the control of constitutive promoters therefore, over, are usually absent in the recipient crops themselves or perform in different pathways. On the other hand, the functions of endogenous proteins are redundant and replaceable in complex networks. Therefore, overexpression of endogenous genes is difficult to cause a significant improvement of phenotypes as the heterologous expression of exogenous genes (Table 2).
- (2) RNAi triggered by antisense or double-stranded RNAs described by transformed synthetic DNA sequences is a

versatile, effective, safe, and eco-friendly technology for crop protection against viruses and other pathogens as well as insect pests, and delaying maturation of fruits with positive economic, environmental, and human health implications (Table 2).

- (3) CRISPR/Cas has developed as the most promising tool for crop improvement. Up to now, several events developed have skipped regulation of government and entered the market because of their safety assurance, and some more events have been in the pipeline of safety assessment (Table 2).

Author contributions

HY drafted the manuscript. QY was responsible for data statistics. FF and WL conceived and supervised the research. All authors interpreted and discussed the data.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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